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(21) International Application Number: PCT/US93/03337 (22) International Filing Date: 9 April 1993 (09.04.93) (30) Priority data: 07/873,330 24 April 1992 (24.04.92) US (71) Applicant: BECKMAN INSTRUMENTS, INC. [US/US]; 2500 Harbor Boulevard, Fullerton, CA 92634 (US). (72) Inventors: REDDY, Parameswara, Meda ; 219 Valverde Avenue, Brea, CA 92621 (US). HANNA, Naeem, Botros ; 616 E. Imperial Hwy., #C, Fullerton, CA 92635 (US). (74) Agent: MAY, William, H.; Beckman Instruments, Inc., 2500 Harbor Boulevard, Fullerton, CA 92634 (US).		(81) Designated States: JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i>
(54) Title: PROTECTING GROUPS USEFUL IN OLIGONUCLEOTIDE SYNTHESIS (57) Abstract Disclosed herein are protecting groups for exocyclic amino groups of the bases adenine, guanine and cytosine for use in the synthesis of oligonucleotides, the protecting groups being represented by the formula: $-\text{CO}-(\text{CH}_2)_{0-9}-\text{CH}_3$. In a particularly preferred embodiment, the base cytosine is protected with acetyl ($-\text{CO}-\text{CH}_3$), and the oligonucleotide incorporating said protected cytosine is subjected to a cleavage/deprotection reagent comprising at least one straight chain alkylamine having from 1 to about 10 carbon atoms.		

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PROTECTING GROUPS USEFUL
IN OLIGONUCLEOTIDE SYNTHESIS

By:

5 Parameswara Meda Reddy and Naeem Botros Hanna

RELATED APPLICATION

10 This application is related to United States
Serial Number _____ (Beckman Docket No.
128D-111), entitled "Methods and Reagents for Cleaving
and Deprotecting Oligonucleotides" by Parameswara Meda
Reddy and Naeem Botros Hanna, which is being filed
15 simultaneously herewith. The related application is
incorporated herein by reference.

FIELD OF INVENTION

20 The present invention is generally directed to
the synthesis of nucleic acids and in particular to
protecting groups useful in the synthesis of nucleic
acids.

BACKGROUND OF THE INVENTION

25 Deoxyribonucleic acid ("DNA") and ribonucleic
acid ("RNA") are long, threadlike macromolecules, DNA
comprising a chain of deoxyribonucleotides, and RNA
comprising a chain of ribonucleotides. A nucleotide
30 consists of a nucleoside and one or more phosphate
groups; a nucleoside consists of a nitrogenous base
linked to a pentose sugar. Typically, the phosphate
group is attached to the fifth-carbon ("C-5") hydroxyl
group ("OH") of the pentose sugar; however, it can also
35 be attached to the third-carbon hydroxyl group ("C-3

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OH"). In a molecule of DNA, the pentose sugar is deoxyribose, while in a molecule of RNA, the pentose sugar is ribose. The nitrogenous bases in DNA are adenine ("A"), cytosine ("C"), guanine ("G") and thymine ("T"). These bases are the same for RNA, except that uracil ("U") replaces thymine. Accordingly, the major nucleosides of DNA, collectively referred to as "deoxynucleosides", are as follows: deoxyadenosine ("dA"); deoxycytidine ("dC"); deoxyguanosine ("dG"); and thymidine ("T"). The corresponding ribonucleosides are designated "A"; "C"; "G"; and "U". (By convention, and because there is no corresponding thymidine ribonucleoside, deoxythymidine is typically designated as "T"; for consistency purposes, however, thymidine will be designated as "dT" throughout this disclosure).

The sequence of the nitrogenous bases of the DNA and RNA molecule encodes the genetic information contained in the molecule. The sugar and phosphate groups of a DNA or RNA molecule perform a structural role, forming the backbone of the molecule. Specifically, the sugar moiety of each nucleotide is linked to the sugar moiety of the adjacent nucleotide such that the 3'-hydroxyl of the pentose sugar of one nucleotide is linked to the 5'-hydroxyl of the pentose sugar of the adjacent nucleotide. The linkage between the two pentose sugars is typically via a phosphodiester bond. Based upon this linkage protocol, one end ("terminus") of the nucleotide chain has a 5'-terminus (e.g. hydroxyl, triphosphate, etc.), and the other end has a 3'-hydroxyl group. By convention, the base sequence of a nucleotide chain is written in a 5' to 3' direction, i.e., 5'-ATCG-3', or, simply ATCG.

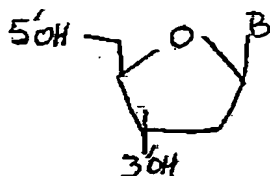
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While DNA and RNA are produced internally by living animals, DNA and RNA can be chemically synthesized such that synthetic strands of DNA and RNA can be rapidly and effectively produced. These strands are typically referred to as "synthetic oligonucleotides" or "oligonucleotides". A widely utilized chemical procedure for the synthesis of oligonucleotides is referred to as the "phosphoramidite methodology". See, e.g., U.S. Patent No. 4, 415,732; McBride, L. and Caruthers, M. Tetrahedron Letters, 24:245-248 (1983); and Sinha, N. et al. Nucleic Acid Res; 17:4539-4557 (1984), which are all incorporated herein by reference. Commercially available oligonucleotide synthesizers based upon the phosphoramidite methodology include, e.g., the Biosearch 8750™ and ABI 380B™, 392™ and 394™ DNA synthesizers.

The importance of chemically synthesized oligonucleotides is principally due to the wide variety of applications to which oligonucleotides can be directed. For example, oligonucleotides can be utilized in biological studies involving genetic engineering, recombinant DNA techniques, antisense DNA, detection of genomic DNA, probing DNA and RNA from various systems, detection of protein-DNA complexes, detection of site directed mutagenesis, primers for DNA and RNA synthesis, primers for amplification techniques such as the polymerase chain reaction, ligase chain reaction, etc, templates, linkers, and molecular interaction studies.

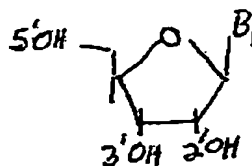
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The primary structures of DNA and RNA molecules can be depicted as follows:

DNA

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10 B= adenine, thymine
guanine, cytosine

RNA

B₁= adenine, uracil
guanine, cytosine

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The key step in nucleic acid synthesis is the specific and sequential formation of internucleotide phosphate linkages between a 5'-OH group of one nucleotide and a 3'-OH group of another nucleotide. Accordingly, in the typical synthesis of oligonucleotides, the phosphate group of an "incoming" nucleotide is combined with the 5'-OH group of another nucleotide (i.e. the 5'-OH group is "phosphorylated" or "phosphitylated"). These groups must be capable of actively participating in the synthesis of the oligonucleotide. Thus, the 5'-OH groups are modified (typically with a dimethoxy trityl ("DMT") group) such that an investigator can introduce two such nucleotides into a reaction chamber and adjust the conditions therein so that the two nucleotides are properly combined; by a series of successive such additions, a growing oligonucleotide having a defined sequence can be accurately generated.

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The four bases of the nucleosides, adenine, thymine (uracil in the case of RNA), guanosine and cytosine, include moieties which are chemically reactive (e.g., exocyclic amino groups). These groups, unlike the

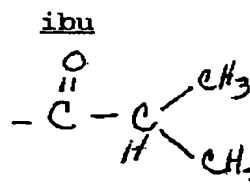
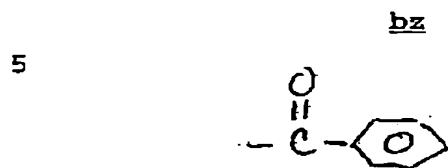
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3'-OH and 5'-OH groups, must be "temporarily" protected, i.e. the protecting groups must be capable of blocking any reactive sites on the base until after the oligonucleotide synthesis is completed; after such synthesis is completed, these groups must also be capable of being removed from the bases such that the biological activity of the oligonucleotide is not affected.

The principal reason for temporarily protecting the base is that in the absence of such protecting groups, the exocyclic amino groups ("NH₂") of the bases can compete for binding to the 5'-OH group. If such a reaction takes place, the resulting product will not be useful. Accordingly, these protecting groups are important in reducing the occurrence of "side product formation" i.e. the formation of chemically similar, but unwanted, materials. Cytidine is particularly susceptible to side product formation during oligonucleotide cleavage and deprotection (i.e. the processes of removing an oligonucleotide from a solid support and removing such protecting groups, respectively). The most widely used protecting groups used in conjunction with the phosphoramidite methodologies for oligonucleotide synthesis are benzoyl for A and C, and isobutyryl for G and C, (thymine, which does not have an amino group, does not ordinarily require a protecting group). By convention, benzoyl is designated "bz", and isobutyryl is designated "ibu", such that the deoxynucleosides protected therewith are typically designated as follows: dA^{bz}; dC^{bz}; dG^{ibu}; and dT^{ibu}.

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Benzoyl and isobutyryl have the following structures:



- 10 Beneficially, these protecting groups can be removed from the oligonucleotide with ammonia (i.e., "deprotected"). Additionally, ammonia can be used to remove oligonucleotides from the solid support material from which they were synthesized (i.e., "cleavage").
- 15 Advantageously, ammonia can be used as a cleavage/deprotection reagent with limited side product formation.

- 20 A practical concern exists, however, with respect to the use of ammonia as a cleavage and deprotection reagent. Ammonia requires a (relatively) long time period to complete the cleavage and deprotection process. On average, 6 minutes is required for the chemical synthesis of each nucleoside to a
- 25 growing oligonucleotide; thus, for an average oligonucleotide of about 21 nucleotides (referred to as a "21-mer"), one can expect that the synthesis will require about 2 hours, using commercially available DNA synthesizers. However, approximately 24 hours (room
- 30 temperature) to 6 hours (55°C) are required for cleavage and deprotection of the oligonucleotide using ammonia. Clearly, more time is required for the final steps of cleavage and deprotection than the synthesis itself. As
- 35 such, an ongoing need has existed for cleavage and deprotection reagents which can complete these steps

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within the same approximate order of magnitude as the synthesis itself. Such reagents are disclosed in the related application referenced above, which is incorporated herein by reference.

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Broadly, such reagents comprise at least one straight chain alkylamine comprising from 1 to about 10 carbon atoms (such an alkylamine can be represented as follows: $\text{-NH}_2(\text{CH}_2)_{0-10}\text{-CH}_3$). In a particularly preferred embodiment of the reagent disclosed in the above-referenced application, a reagent comprising methylamine and t-butylamine can be utilized to cleave and deprotect oligonucleotides in less than about 90 minutes at room temperature, and less than about 10 minutes at about 65°C.

20

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It was observed that when these reagents are used in conjunction with oligonucleotides comprising dC^{bz} or dC^{ibu} , the formation of an unwanted side product, N-methylcytidine, could occur. With respect to dC^{bz} , approximately 10% of the cytidines within the oligonucleotides were N-methylcytidine. Thus, while on the one hand a cleavage/deprotection reagent which could rapidly accomplish these tasks had been discovered, on the other hand such reagent, when used in conjunction with the so called "traditional" bz and ibu protecting groups for the base cytidine, led to cytidine side product formation.

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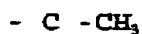
What is needed, then, are protecting groups useful in oligonucleotide synthesis which do not have such deleterious side effects.

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SUMMARY OF THE INVENTION

Disclosed herein are protecting groups which satisfy at least the above need. The disclosed protecting groups, which have the following characteristics and are thus broadly defined thereby are at least about 30 times more labile than benzoyl, and comprise a carbonyl group having a straight chain alkyl group attached thereto, the alkyl group comprising from 1 to about 10 carbon atoms, preferably from about 1 to about 6 carbon atoms, more preferably from 1 to about 3 carbon atoms, and most preferably 1 carbon atom. A particularly preferred protecting group is acetyl, represented by the following formula:



and designated herein as "Ac". Most preferably, the disclosed protecting group is used to protect cytidine bases; Ac protected deoxycytide is designated herein as "dC^{Ac}".

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings are intended to be used for purposes of illumination of the Detailed Description of Preferred Embodiments:

Fig. 1 is a schematic representation of the chemical synthesis disclosed in Examples I - IV;

Fig. 2 is a photographic reproduction of a polyacrylamide gel electrophoresis analysis of various 35-, 51- and 101-mers comprising various percentages of dC^{Ac} and dC^{Hz} and subjected to either methylamine/t-butylamine or ammonia cleavage/deprotection reagents;

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Fig. 3 is an electropherogram of a heterogeneous 51-mer comprising 35% dC^{bz} subjected to ammonia as a cleavage/deprotecting reagent;

5 Fig. 4 is an electropherogram of a heterogeneous 51-mer comprising 35% dC^{Ac} subjected to methylamine/t-butylamine as a cleavage/deprotection reagent;

10 Fig. 5 is a photographic reproduction of PCR-derived 957 base-pair amplified template;

15 Fig. 6 is a photographic reproduction of a sequencing reaction of the template M13mp18;

20 Fig. 7 is an electropherogram of a 3'-Terminal Transferase extension initiated using a 22-mer comprising dC^{Ac} and subjected to methylamine/t-butylamine as a cleaving/deprotecting reagent; and

25 Fig. 8 is an electropherogram of a 3'-Terminal Transferase extension initiated using a 22-mer comprising dC^{bz} and subjected to ammonia as a cleaving/deprotecting reagent.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

30 As those in the art appreciate, the base cytidine is most susceptible to side product formation during the deprotection of oligonucleotides. Thus, by convention, it is useful to monitor side product formation of cytidine during oligonucleotide synthesis and deprotection.

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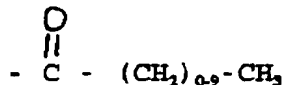
During the course of investigating cleavage/deprotection reagents comprising a straight chain alkylamine having from between 1 to about 10 carbon atoms, it was discovered that oligonucleotides containing a cytidine base protected by benzoyl ("bz") or isobutyryl ("ibu") and which were subjected to such reagents had some cytidine side product formation, specifically in the form of N-methylcytidine. Accordingly, while such reagents provide the ability to rapidly cleave and deprotect oligonucleotides compared to, inter alia, ammonia, the resulting side product formation led to the need for a different protecting group for cytidine bases which would not lead to such side product formation. Such protecting groups require at least the following criteria: susceptible to deprotection comparable to bz and ibu, and not lead to the formation of statistically significant side product formation (i.e., average less than about 0.01%). Additionally, the resulting oligonucleotide must retain, its biological activity. I.e., the oligonucleotide must be useful in terms of, complementary base pairing between, e.g., the bases C and G.

The present invention is a protection group comprising a carbonyl group having a straight chain alkyl group attached thereto, the alkyl group having from 1 to about 10 carbon atoms, preferably from 1 to about 6 carbon atoms, more preferably from 1 to about 3 carbon atoms, and most preferably 1 carbon atom. When the present invention is used in conjunction with a cleavage/deprotection reagent comprising at least one straight chain alkylamine having from between 1 to about 10 carbon atoms, it results in significantly reduced cytidine side product formation.

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As used herein, the term "labile" means the capability of undergoing a chemical change. The protecting groups of the present invention can be represented as follows:

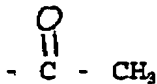
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As used herein, the term "oligonucleotide" is meant to encompass synthetic oligonucleotide as well as modified oligonucleotides, i.e. where the 3' end, 5' end, sugar, or heterocyclic base are modified, as well as modification of the phosphate backbone (e.g. methyl phosphonates, phosphorothioates, and phosphoramidates). Additionally, oligonucleotides can also include oligonucleotides having an attached reporter group, e.g. biotin, avidin, haptens, dyes, fluorescent, chemiluminescent, enzymatic or radioactive labels, and solid supports other than the solid support from which the oligonucleotide is synthesized.

A particularly preferred protecting group in accordance with the disclosure is acetyl, represented as follows:

25



and referenced herein as "Ac". Thus, deoxycytidine protected with acetyl is designated herein as "dC^{Ac}".

30

While not wishing to be bound to any particular theory, it is believed that the relative lability of bz versus ibu is responsible for an increase in cytidine side product formation in the presence of an alkylamine cleavage/deprotection reagent. Ibu is (relatively) more

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labile than bz; in the presence of an alkylamine, oligonucleotides containing cytidine base protected with ibu led to less percentage of N-methylcytidine side product formation than comparative oligonucleotides containing cytidine protected with bz. Accordingly, it was postulated that chemical moieties which could function as protecting groups and which were more labile than bz might evidence statistically less cytidine side product formation. It was further postulated that lability is affected by the electronic donation of the groups adjacent to the carbonyl group. I.e., as the electronic donation increases, the carbonyl carbon becomes less electropositive and hence less susceptible to nucleophilic attack by the deprotection reagent. For example, the electron donation from the secondary (i.e., branched) carbons to the carbonyl carbon of ibu is greater than the donation from the primary carbon to the carbonyl carbon of Ac.

Acetyl is significantly more labile than ibu and, additionally, is the least "bulky" of the disclosed, defined protecting groups. Acetyl can be readily conjugated to the exocyclic amino group of the cytidine base, and, as determined experimentally, can be efficiently and effectively utilized in conjunction with inter alia, alkylamine cleavage/deprotection reagents without statistically significant side-product formation.

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EXAMPLES

The following Examples, directed to preferred embodiments of the invention are not intended, nor should they be construed that be, limitations on the disclosure or the claims that follow:

I. Materials and Methods

A. Reagents

1. Cleavage/Deprotection Reagent

All chemicals were at least of ACS grade. Ammonium hydroxide was obtained from Aldrich (Milwaukee, WI.; Cat. No. 22, 122-8). Methylamine, 40 wt% solution in water, was obtained from Aldrich (Cat. No. M2, 775-1), as was t-butylamine (Cat. No. B8, 920-5).

Methylamine/t-butylamine reagent was prepared by mixing a 1:1 volume-to-volume ratio, followed by shaking for 5 minutes at room temperature and storage at 4°C. Ammonium hydroxide was stored in accordance with supplier instructions.

2. Protected Deoxynucleosides

The following protected deoxynucleosides were obtained from Sigma Chemical Co. (St. Louis, Mo.):

- a) dA^{bz} (Cat. No. B 6130);
- b) dC^{bz} (Cat. No. B 5882);
- c) dC^{tbu} (Cat. No. I 6261); and
- d) dG^{tbu} (Cat. No. I 6007).

Thymidine was obtained from Sigma (Cat. No. T 5018).

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B. Commercially Available Protocols**1. Polymerase Chain Reaction ("PCR")**

5 PCR analysis of oligonucleotide primers
subjected to the disclosed cleavage/deprotection reagent
was conducted using a Perkin Elmer Cetus GeneAmp™ DNA
Amplification Reagent Kit with AmpliTag™ (Part NO. N801-
0055). Manufacturer instructions were followed.

2. DNA Sequencing

10 Sequencing reaction was performed using M13mp18
single stranded DNA template (New England Biolabs, Cat.
15 No. 404-C) following the protocol of United States
Biochemical Sequenase® Version 1.0, using α -[³⁵S]-dATP.

C. Instruments**1. Automated DNA Synthesizer**

20 Synthesis of oligonucleotides was performed
using a Biosearch 8750™ DNA synthesizer; controlled pore
glass (CPG), 500Å-1000Å pore size, was used for the solid
25 support material. Homo- and hetero-oligonucleotides of
various lengths were synthesized in accordance with
manufacturer instructions.

2. Capillary Electrophoresis

30 Capillary electrophoresis of oligonucleotides
was performed on a Beckman Instruments, Inc. P/ACE™ 2000
high performance capillary electrophoresis system. A
37cm U100P Urea Gel Column (Beckman, Cat. No. 338480) was
35 utilized. Samples were loaded onto the columns via the

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electrokinetic injection method (10kV, 3.seconds); separation was conducted at 11kV/cm for 30-90 minutes, depending on oligonucleotide length. Tris-hydroxymethyl aminomethane ("TRIS")-borate 7M urea running buffer (Beckman, Gel Buffer Kit, Cat. No. 338481) was utilized. Absorbance detection was in the range of from 0.05 to 2.0 OD_{260nm}/ml, depending principally on the length of the oligonucleotide.

3. High Pressure Liquid Chromatography ("HPLC")

HPLC analysis was conducted on a Beckman Instruments System Gold™ HPLC Programmable Solvent Module 126 equipped with a diode array detector module 168 and autosampler 507. A C₁₈ Ultrasphere™ HPLC column (Beckman, Cat. No. 235329; 5μ particles, 4.6mm x 25cm) was utilized. Bottle A contained 0.1M ammonium acetate, pH 6.9; Bottle B contained HPLC-grade acetonitrile. The system was operated in a gradient mode as follows (1ml/min. flow rate): 0-10 min: 85% Bottle A, 15% Bottle B; 20-25 min: 75% Bottle A, 25% Bottle B; 25-27 min: 50% Bottle A, 50% Bottle B; 27-30 min: 50% Bottle A, 50% Bottle B; 30-35 min, 100% Bottle A, 0% Bottle B.

II. Example I. Preparation of 2' Deoxycytidine

A suspension of 71g (269.3 mmol) of 2'-deoxycytidine - hydrochloric acid (Pennisula, Belmont, CA.; Cat. No. N1012) and 1600ml methylene chloride was admixed with 42ml (301mmol) triethylamine (Aldrich; Cat. No. 206-3). The admixture was vigorously stirred at ambient temperature for 4 hrs. A colorless, crystalline solid was collected, washed with methylene chloride (3X80ml) and air dried. 61g (99% yield) of a material having a melting point within the range of 185°-195°C was

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obtained; the published melting point of free base 2-deoxycytidine is 185°-195°C.

Example II. Preparation of N⁴-Acetyl-2'-Deoxycytidine

To 61.29g (270mmol) of the material of Example I dissolved in 1300ml of anhydrous N,N-dimethylformamide ("DMF") (Aldrich; Cat. No. 22, 70506), was added 28ml (296mmol) of acetic anhydride (Aldrich; Cat. No. 11,004-3), and the resulting mixture was stirred at room temperature for 20 hrs. DMF was removed under reduced pressure, and the resulting residue was treated with the excess of 100ml dimethyl ether; 71.4g (98% yield) of a crystalline product was obtained and collected by filtration, washed thoroughly with dimethyl ether, and dried over P₂O₅ for 3 hrs. This product had a melting point of 150-170°C; the published melting point for this product 154-176°C.

The calculated compositional molecular weight for N⁴-acetyl-2'-deoxycytidine-H₂O (C₁₁H₁₅N₃O₅-H₂O) is: C-45.99; H-5.97; and N-14.63. The crystalline product had the following compositional molecular formula as determined by elemental analysis: C-45.71; H-6.10; and N-14.38. It was further determined by infra-red spectra that the crystalline product contained a single carbonyl amide moiety and a single carbonyl ring amide. The structure was further confirmed by nuclear magnetic resonance ("NMR"). The foregoing is consistent with the structure of N⁴-acetyl-2'-deoxycytidine.

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Example III. Preparation of N⁴-Acetyl-5'-O-(4,4'-dimethoxy-trityl)-2'-Dexoycytidin

70g (260.2mmol) of the product of Example II was dried by co-evaporation with 2X50ml dry pyridine (Aldrich; Cat. No. 27,097-0), then taken up in 1300ml of dry pyridine, ice-cooled; thereafter, 105g (314mmol) of 4,4'-dimethoxy-trityl chloride ("DMTr-Cl") (Peninsula; Cat. No. N4011) was added to the solution. The mixture was left stirring at 5°C for 20 hrs. Pyridine was removed under reduced pressure, and the resulting residue was taken up in 3.0 liters of methylene chloride, washed with 2X2 liters of 5% sodium hydrogen carbonate (Aldrich; Cat. No. 23,931-3) and 1X2 liters of deionized water. The organic layer was dried over sodium sulfate and concentrated to near dryness. The product was purified on a 6X80cm silica gel column (Aldrich; 70-230 mesh; Cat. No. 28,864-4) by gradient elution with 20.0 liters 0-6% methylene-chloride-methanol. Desired fractions were collected, concentrated to approximately 300ml, and added drop-wise to 3.0 liters cooled (0°C) hexane (Baxter, McGaw Port, IL.; Cat. No. 216-4 DK) to precipitate the product. The precipitated product was filtered, washed with hexane and air dried to yield 117g (79% yield) of a product.

The calculated compositional molecular weight for N⁴-acetyl-5'-O-(4,4'-dimethoxy-trityl)-2'-deoxycytidine (C₃₂ H₃₃ N₃ O₇) is: C-67.24; H-5.82; and N-7.35. The product had the following compositional molecular formula as determined by elemental analysis: C-66.02, H-6.05; and N-6.91. It was further determined by infra-red spectra that the crystalline product contained a single carbonyl amide moiety and a single carbonyl ring amide. The structure was further confirmed by NMR. The

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foregoing is consistent with the structure of N⁴-acetyl-5'-O-(4,4'-dimethoxy-trityl)-2'-deoxycytidine.

5 **Example IV.** Preparation of N⁴-Acetyl-5'-O-(4,4'-dimethoxy-trityl)-2'-deoxycytidine-3'-O-(N,N-diisopropyl)-β-cyanoethyl-phosphoramidite.

10 11.44g (20mmol) of the product of Example III was dried by successive co-evaporation with pyridine, toluene and tetrahydrofuran ("THF") (Aldrich; Cat. No. 18,656-2), refluxed and distilled over CaH₂. The dried residue was dissolved in 100ml of dry THF and 14ml (80mmol) of N,N,N-diisopropylethylamine was added
15 thereto. This was followed by 5 min drop-wise addition (via a syringe) of 8.92ml (40mmol) β-cyanoethylmonochloro-N,N-diisopropyl phosphoramidite with constant stirring under argon at room temperature. After 60 min of stirring, 1.2ml of methanol (40mmol) was added to the
20 mixture, and stirring was continued for another 60 min, and evaporated to dryness. The residue was dissolved in 600ml ethylacetate (Baxter; Cat. No. CP80132-4DK), washed with 10% NaHCO₃ (2X500ml) and dried over Na₂SO₄. The organic layer was evaporated and the residue was
25 dissolved in 50ml ether; this was then added by drop-wise addition to 700ml of hexane at room temperature. The mixture was decanted, and the precipitated product was dissolved in 100ml ether, followed by the addition of 700ml of hexane and stirring at room temperature. This
30 mixture was decanted and the product dissolved in 500ml CH₂Cl₂, followed by the addition of 30g basic alumina (Aldrich; Cat. No. 19,944-3) and stirring for 1 hr at room temperature. The mixture was filtered in a sintered glass funnel, evaporated and dried in a desiccator over
35 CaCl₂, P₂O₅ under reduced pressure. 11g (76% yield) of a

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product was obtained; as determined by reverse-phase HPLC, the purity thereof was 98.4%.

The calculated compositional molecular weight for N⁴-acetyl-5'-O-(4,4'-dimethoxy-trityl)-2'-deoxycytidine-3'-O-(N,N-diisopropyl)-β-cyanoethyl-phosphoramidite (C₄₁H₅₀N₅O₈P) is: C-63.80; H-6.53; N-9.07; and P-4.01. The compositional molecular formula of the product, as determined by elemental analysis, was: C-62.51; H-6.84; N-8.68, and P-3.61. It was further determined by infra-red spectra that this product contained a single carbonyl amide moiety, a single carbonyl ring amide and a single -C-N group. The structure was further confirmed by NMR. The foregoing is consistent with the structure of N⁴-acetyl-5'-O-(4,4'-dimethoxy-trityl)-2'-deoxycytidine-3'-O-(N,N-diisopropyl)-β-cyanoethyl-phosphoramidite.

The product in Example IV was designated "dC^{Ac}" as indicating a deoxycytidine comprising an acetyl protecting group. A schematic diagram outlining the preparation steps of Examples I - IV is set forth in Figure 1.

Example V: Cytidine Side Product Formation

As noted, deoxycytidine is ordinarily most susceptible to side product formation during deprotection of oligonucleotides comprising deoxycytidine. Typically, such side product formation is via transamination.

As those in the art appreciate, the synthesis of oligonucleotides is typically conducted with the intent of retrieving the end-product as quickly as possible. Occasionally, however, it is possible that the

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solubilized, deprotected oligonucleotide may remain in a deprotection reagent for extended time periods. As those in the art further appreciate, such an increase in time when the oligonucleotide is within the reagent can increase the chance of a transamination event, thus increasing the chance of side product formation.

Cytidine side product formation was investigated by reverse phase HPLC, using both methylamine and methylamine/t-butylamine as the reagent, across several times and temperatures. The "traditional" cytidine protecting groups, "bz" and "ibu" were studied, as well as an acetyl protecting group. The side product observed when utilizing such reagent (as confirmed by Nuclear Magnetic Resonance) was N-methyl cytidine. Percentage of N-methyldeoxycytidine formation, relative to deoxycytidine, are presented below, based upon solution-based deprotection of deoxycytidine protected with an acetyl protecting group:

TABLE I

Percentage of N-methylcytidine Formation*

Reagent	TEMPERATURE		
	25°C	37°C	65°C
Methylamine	<0.01** (60 min.)	<0.01 (20 min.)	<0.01 (5 min.)
Methylamine (16 hrs.)	~0.05	~0.25	~2.5
Methylamine/ t-butylamine (16 hrs.)	<0.01	<0.01	~0.6%

* - average percentages

** - 0.01% is the lowest detectable limit of the instrument

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These results indicate that for a typical oligonucleotide synthesis (i.e. one in which the investigator is desirous of obtaining the finished end product as soon as possible), the methylamine does not lead to statistically significant cytidine side product formation. However, as the time that the oligonucleotide remains in the reagent increases, so too does the formation of cytidine side product formation. Thus, the use of a Transamination Suppression Reagent ("TSA") is useful. A "TSA" is defined herein as an agent useful in the suppression of transamination, i.e. the exchange of amines on a nucleotide, typically manifested as side-product formation. Preferably, the TSA is an agent (or agents) having a polarity index value that is at least 1.5 times less than that for water, preferably selected from the group consisting of straight chain, branched, cyclic, saturated and unsaturated alkylamines having from between 1 and about 10 carbon atoms and which may further comprise functional groups; ethanol; methanol; isopropylamine; acetyl nitrile; dimethylformamamide; tetrahydrofuran; and combinations of the foregoing. Exemplary alkylamines as defined include, but are not limited to, t-butylamine, ethylamine, propylamine, isopropylamine, dimethylamine, diethylamine, trimethylamine and secondary butylamine. The data indicates that relative to methylamine, a reagent comprising methylamine and t-butylamine as a TSA significantly reduces cytidine side product formation.

A secondary set of studies was conducted along these lines. For these studies, side product formation for dC^{AC}, dC^{bu}, dC^{tz}, dG^{bu}, dA^{tz} and dT (as a relative percentage of non-side product formation for the nucleosides) were investigated at various times and

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temperatures using methylamine/t-butylamine as the reagent. Results are as presented in Table II:

=====

5

TABLE II

Percentage of Side Product Formation*

	Temp. (°C)	Reaction Time	C ^{AC}	C ^{ibu}	C ^{bz}	G ^{ibu}	A ^{bz}	T
10	25	90 min.	**	0.15	10.0	**	**	**
	25	16 hrs.	**	***	***	**	**	**
	37	30 min.	**	0.15	10.0	**	**	**
	37	5 hrs.	**	***	***	**	**	**
	37	16 hrs.	**	***	***	**	**	**
15	65	5 min.	**	0.15	10.0	**	**	**
	65	1 hr.	**	***	***	**	**	**
	65	16 hrs.	0.6	***	***	**	**	**
	80	3 min.	**	0.15	10.0	**	**	**
	80	1 hr.	***	***	**	**	**	**

20

* Averages

** <0.01

*** not investigated due to high percentages
at optimal temperature/reaction time

25

=====

These results indicate at least several things. First, with respect to the dC protection groups, the data indicates that acetyl protecting groups provide superior results when used in conjunction with the straight chain alkylamine cleaving and deprotecting reagent; the "traditional" cytidine protecting groups resulted in significantly higher side product formation. Second, this deprotecting and cleaving reagent does not lead to statistically significant side product formation for any of the protected deoxynucleosides at any of the

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investigated temperatures or reaction times, with the exception of deprotection of dC^{Ac} at the elevated temperatures and at times greater than the desired reaction times. Thus, for oligonucleotides comprising deoxycytidine protected with an acetyl protecting group, it is preferred that at such elevated temperatures, extended reaction times not be utilized.

Example VI: Enzymatic Digestion Analysis of Non-Purified Oligonucleotides

Analysis of the composition of several oligonucleotides were conducted using enzymatic digestion and reverse phase HPLC techniques. These studies were conducted using deoxycytidines protected with an acetyl protecting group and a traditional protecting group, bz; all other protecting groups were consistent between the oligonucleotides. 35-mers, 51-mers and 101-mers, having the following sequences, were analyzed:

35-mer

5' - CAG-TGC-AGC-TCC-TAG-CAG-CCT-AGC-GTA-CTA-GTC-TT-3'

51-mer

5' - CAG-TCC-TAG-TCA-CAG-TCC-AGT-CGC-TCA-AGC-GTC-CAG-TTG-CAC-AGG-TCA-CCT-3'

101-mer

5' - GCT-GCC-AGT-TCG-GTC-ATC-CGA-TCC-TCG-GTC-ACG-CAA-CTG-TCA-ACG-GCA-CCT-ACT-CCT-CGT-AAC-GTA-GGA-CAG-TCC-GAT-TCG-C4C-GTG-CAA-AGC-CCA-TTC-AT-3'

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The oligonucleotides were cleaved and deprotected using a reagent comprising methylamine/t-butylamine at 25°C for 90minutes or ammonia for 3hrs. at 65°C; solubilized, deprotected oligonucleotides were not purified prior to analysis. Results are as follows in Table III:

TABLE III
Composition Analysis

		Determined		
		Theoretical	Oligonucleotides Comprising dC ^{As}	Oligonucleotides Comprising dC ^{As}
35-mer	C	11	10.67	10.62
	G	8	7.88	7.84
	T	9	9.65	9.41
	A	7	6.80	7.13
51-mer	C	18	17.04	17.36
	G	11	11.71	11.72
	T	11	11.61	11.07
	A	11	10.65	10.85
101-mer	C	35	33.80	33.53
	G	22	20.74	20.70
	T	22	21.78	21.73
	A	22	25.09	25.04

The theoretical composition of the various non-purified oligonucleotides and the determined composition provide good correlation. Additionally, the difference in deoxycytidine protecting groups, based upon the above

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data, does not indicate a statistically significant difference in results.

Example VII. Polyacrylamide Gel Electrophoresis
("PAGE")

Analysis of 35-mers (35% dC^{bz}; 35% dC^{Ac}; 100% dC^{bz}; and 100% dC^{Ac}), 51-mers (35% dC^{bz}; 35% dC^{Ac}; 100% dC^{bz}; and 100% dC^{Ac}); and 101-mers (35% dC^{bz}; 35% dC^{Ac}; 100% dC^{bz}; and 100% dC^{Ac}) were analyzed by PAGE. The hetero 35-, 51- and 101-mers were as described in Example IV and for the homo 35-, 51- and 101-mers, the oligomer was synthesized from an insolubilized thymidine. Oligonucleotides comprising dC^{Ac} were cleaved and deprotected using a reagent comprising methylamine/t-butylamine for 90min. at 65°C; oligonucleotides comprising dC^{bz} were cleaved and deprotected using ammonia for 3hrs. at 65°C.

A 22cm x 16.5cm denaturing gel was prepared by adding 107.3ml of deionized water to 100gm of premixed acrylamide/methylene bis-acrylamide (29:1) (Boehringer Mannheim Biochemicals, Indianapolis, IN; Cat. No. 100-151) to achieve a 50% stock solution. To 20ml of the 50% stock solution was added 22.5g urea, 5ml of 10 x Tris-Borate/EDTA ("TBE") and sufficient deionized water to achieve 50 ml. The solution was stirred and heated such that the solid constituents were dissolved. Thereafter, 20mg ammonium persulfate and 20μl N,N,N',N'- Tetra-methylethylene diamine ("TEMED") was added; this solution was poured into clean plates and allowed to polymerize for 1 hr. gels when pre-run with 1 x TBE at 20mA for 1 hr. 0.2-1.0 OD_{260nm} of each oligonucleotide was added to 10μl of 10m urea. The 20μl admixtures were loaded onto the gel and electrophoresed at 28mA for 2-4 hours, depending on the length of the oligonucleotide. Bands

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were visualized by UV shadowing on TLC fluorescent plate or by ethidium bromide staining.

Photographic results are presented in Figure 2,
5 where the lanes are defined as follows:

	<u>Lane</u>	<u>Oligonucleotide</u>
	1	35-mer (35% dC ^{Ac})
10	2	35-mer (35% dC ^{bz})
	3	35-mer (100% dC ^{Ac})
	4	35-mer (100% dC ^{bz})
	5	51-mer (35% dC ^{Ac})
	6	51-mer (35% dC ^{bz})
15	7	51-mer (100% dC ^{Ac})
	8	51-mer (100% dC ^{bz})
	9	101-mer (35% dC ^{Ac})
	10	101-mer (35% dC ^{bz})
	11	101-mer (100% dC ^{Ac})
20	12	101-mer (100% dC ^{bz})

The results of Figure 2 indicate that the oligonucleotides subjected to methylamine/t-butylamine reagent and Ac protection group provided nearly identical
25 PAGE patterns compared to the oligonucleotides subjected to ammonia and the traditional deoxycytidine protecting group, bz.

Example VIII. Capillary Electrophoresis

30

Heterogeneous 51-mer oligonucleotides comprising either 35% dC^{bz} or 35% dC^{Ac} were subjected to either ammonia for 3hrs. at 65°C or methylamine/t-butylamine for 90min at 25°C, respectively, and were
35 analyzed by capillary electrophoretic techniques.

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Electropherograms for the oligonucleotide subjected to ammonia and a reagent comprising methylamine/t-butylamine are presented in Figure 3 and 4, respectively.

5 The results of Figures 3 and 4 are nearly identical in terms of time from sample introduction to detection of the 51-mer. The percent-of-total integrated areas beneath the major peaks, 66.902 for Figure 3 and 66.575 for Figure 4, are also nearly identical. These
10 results further indicate that the methylamine/t-butylamine reagent and deoxycytidine protecting group Ac provide comparatively identical soluble, deprotected oligonucleotides vis-a-vis ammonia and the traditional deoxycytidine protecting group, bz.

15 **Example X. Polymerase Chain Reaction**

 The foregoing Examples evidences that a deprotection/cleavage reagent comprising a straight chain
20 alkylamine comprising from between 1 and about 10 carbon atoms and acetyl protecting group can be utilized to rapidly and efficiently cleave and deprotect oligonucleotides comprising, inter alia, deoxycytidine, with statistically insignificant side-product formation.
25 As those skilled in the art appreciate, however, it is necessary to be able to utilize such oligonucleotides for a variety of procedures.

 Oligonucleotides used as primers in a
30 polymerase chain reaction where generated and subjected to methylamine/t-butylamine reagent (where the deoxycytidines were protected with Ac) for 90min. at 25°C. The primers were as follows:

35

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Lane 1	957 bp product	(primers derived using methylamine/t-butylamine reagent and acetyl protecting group for deoxycytidine);
Lane 2	957 bp product	(primers derived using ammonia and bz protecting group for deoxycytidine);
Lane 3	Gel Marker	(Lambda DNA digested with Hind III, 2322 and 2027 bp markers); and
Lane 4	Gel Marker	(PBR322 DNA digested with Hinf I, 1632 and 506 bp marker)

5

The results presented in Figure 5 indicate that primers derived utilizing the methylamine/t-butylamine reagent and the acetyl protecting group led to the production of an amplified product substantially identical to that derived from primers generated by ammonia cleavage and deprotection and using a bz protecting group for deoxycytidine.

10

Example XI. DNA Sequencing

15

Two sets of 18-mers were synthesized using an acetyl protecting group for deoxycytide and, for comparative purposes, bz, and were subjected to a reagent comprising methylamine/t-butylamine for 90min. at 25°C, and ammonia for 3hrs. at 65°C, respectively. The 18-mers had the following sequence:

20

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18-mer

5'-CGC-CAG-GGT-TTT-CCC-AGT-3'

5 Solubilized, deprotected oligomers were purified using Sep Pak (Waters, Part no. 5190) DNA purification kit. These purified oligomers were used as primers for sequencing purposes. The template was M13mp18 single stranded DNA (New England Biolabs, Cat. No. 404-C); sequencing was accomplished using the 18-mers in conjunction with, the USB Sequenase materials and protocols. Results are presented in Figure 6.

15 As the results of Figure 6 indicate, the sequencing band patterns are substantially identical using primers subjected to a methylamine/t-butylamine reagent and acetyl protecting group vis-a-vis primers derived via ammonia and bz.

20 **Example XII. 3' Terminal Transferase Extension**

22-mers were synthesized using either an acetyl or bz protecting group for deoxycytidine, and were subjected to a reagent comprising methylamine/t-butylamine for 90min. at 65°C, or ammonia for 4hrs. at 65°C, respectively. The 22-mers had the following sequence:

22-mer

30 5'-TTC-TGC-CGT-ACC-GTT-CCT-GTC-T-3'

Solubilized, deprotected oligomers were purified using Sep Pak DNA purification kit. These purified oligomers

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were used as primers for 3' terminal transferase extension studies.

2.5 OD_{260nm} of each oligonucleotide was added to
5 150 μ l of deionized water; 5mg thymidine triphosphate
("TTP"), (Sigma, Cat. No. T8635); 5 μ l terminal
deoxynucleotidyltransferase ("TDT"), 15U/ μ l (BRL, Cat.
No. 8008SB) and 50 μ l trailing buffer. The admixture was
incubated overnight at 37°C and the resulting material
10 purified using a Sep Pak C₁₈ cartridge as follows: the
reaction mixture was diluted 1:2 in 0.5M ammonium
acetate, loaded onto the cartridge, followed by washing
of the cartridge with deionized water, and the product
eluted with 60% methanol in deionized water. The
15 products were analyzed by capillary electrophoresis;
electropherogram results are presented in Figures 7 and
8.

The electropherograms of Figures 7 and 8
20 evidence that the primers comprising cytidine protected
with acetyl and subjected to a methylamine/t-butylamine
reagent (Fig. 7) and primers comprising cytidine
protected with bz and subjected to ammonia (Fig. 8) were
both extended at the 3' ends thereof, and that the
25 resulting products were substantially identical.

While the foregoing has been described in
considerable detail, it is to be understood that the
embodiments disclosed in the Detailed Description and
30 Examples are not to be construed as limiting to the
disclosure or the claims to follow. The invention is not
limited to automated DNA synthesizers. The invention is
not limited to deoxyribonucleic acid oligonucleotides,
but can also be utilized with ribonucleic acid
35 oligonucleotides. The invention is not limited to the

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use of the disclosed protecting groups only with respect
to the base cytosine. The invention is not limited to
use in conjunction with the specific embodiment of the
reagent disclosed in the referenced co-pending
5 application, but rather is intended to be utilized in
conjunction with inter alia, the reagents broadly
disclosed and claimed therein. Modifications and changes
that are within the purview of those skilled in the art
are intended to fall within the scope of the following
10 claims.

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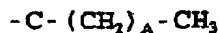
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What is claimed is:

1. An exocyclic amino protecting group useful in the synthesis of oligonucleotides and having the formula:



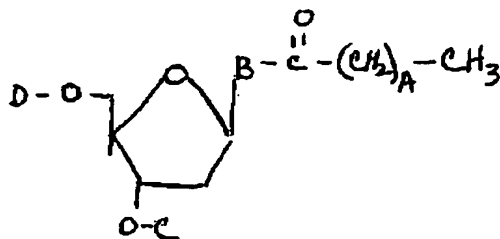
where "A" is a whole number between 0 and about 9.

2. The protecting group of claim 1 wherein A is a whole number between 0 and about 5.

3. The protecting group of claim 1 wherein A is a whole number between 0 and about 2.

4. The protecting group of claim 1 wherein A is 0.

5. A compound having the following structure:



where "A" is a whole number between 0 and about 9; B is selected from the group consisting of adenine, guanine, and cytosine; C is selected from the group consisting of a hydrogen atom, a reporter group, a group capable of binding, during the synthesis of an oligonucleotide, to a 5' carbon atom of a nucleoside, and a group capable of binding, during the synthesis of an oligonucleotide, to an oxygen atom bound to a 5' carbon atom of a nucleoside;

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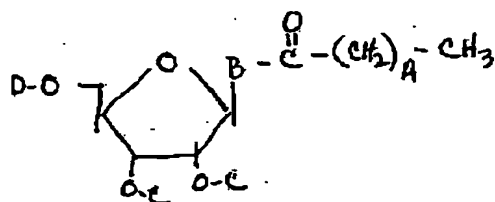
and D is selected from the group consisting of hydrogen, a reporter group, a group capable of binding, during the synthesis of an oligonucleotide, to a 3' carbon atom of a nucleoside, and a group capable of binding, during the synthesis of an oligonucleotide, to an oxygen atom bound to a 3' carbon of a nucleoside.

6. The compound of claim 5 wherein A is a whole number between 0 and 5.

7. The compound of claim 5 wherein A is a whole number between 0 and 2.

8. The compound of claim 5 wherein A is 0.

9. A compound having the following structure:



where "A" is a whole number value between 0 and about 9; B is selected from the group consisting of adenine, guanine, and cytosine; C' is selected from the group consisting of a hydrogen atom, a reporter group, and a group capable of being chemically removed from the 2' oxygen atom and replaced thereby with a hydrogen atom; C is selected from the group consisting of a hydrogen atom, a reporter group, a group capable of binding, during the synthesis of an oligonucleotide, to a 5' carbon atom of a nucleoside, and a group capable of binding, during the synthesis of an oligonucleotide, to an oxygen atom bound to a 5' carbon atom of a nucleoside; and D is

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selected from the group consisting of hydrogen, a reporter group, a group capable of binding, during the synthesis of an oligonucleotide, to a 3' carbon atom of a nucleoside, and a group capable of binding, during the synthesis of an oligonucleotide, to an oxygen atom bound to a 3' carbon of a nucleoside.

10. The compound of claim 9 wherein A is a whole number between 0 and about 5.

11. The compound of claim 9 wherein A is a whole number between 0 and about 2.

12. The compound of claim 9 wherein A is 0.

13. The exocyclic amino protecting group of claim 1 wherein said protecting group is used to protect an exocyclic amino group of a nucleotide used in the synthesis of an oligonucleotide, the oligonucleotide being subjected to a reagent comprising at least one straight chain alkylamine comprising from 1 to about 10 carbon atoms.

14. The compound of claim 5 used in the synthesis of an oligonucleotide, the oligonucleotide being subjected to a reagent comprising at least one straight chain alkylamine comprising from 1 to about 10 carbon atoms.

15. The compound of claim 9 used in the synthesis of an oligonucleotide, the oligonucleotide being subjected to at least one straight chain alkylamine comprising from 1 to about 10 carbon atoms.

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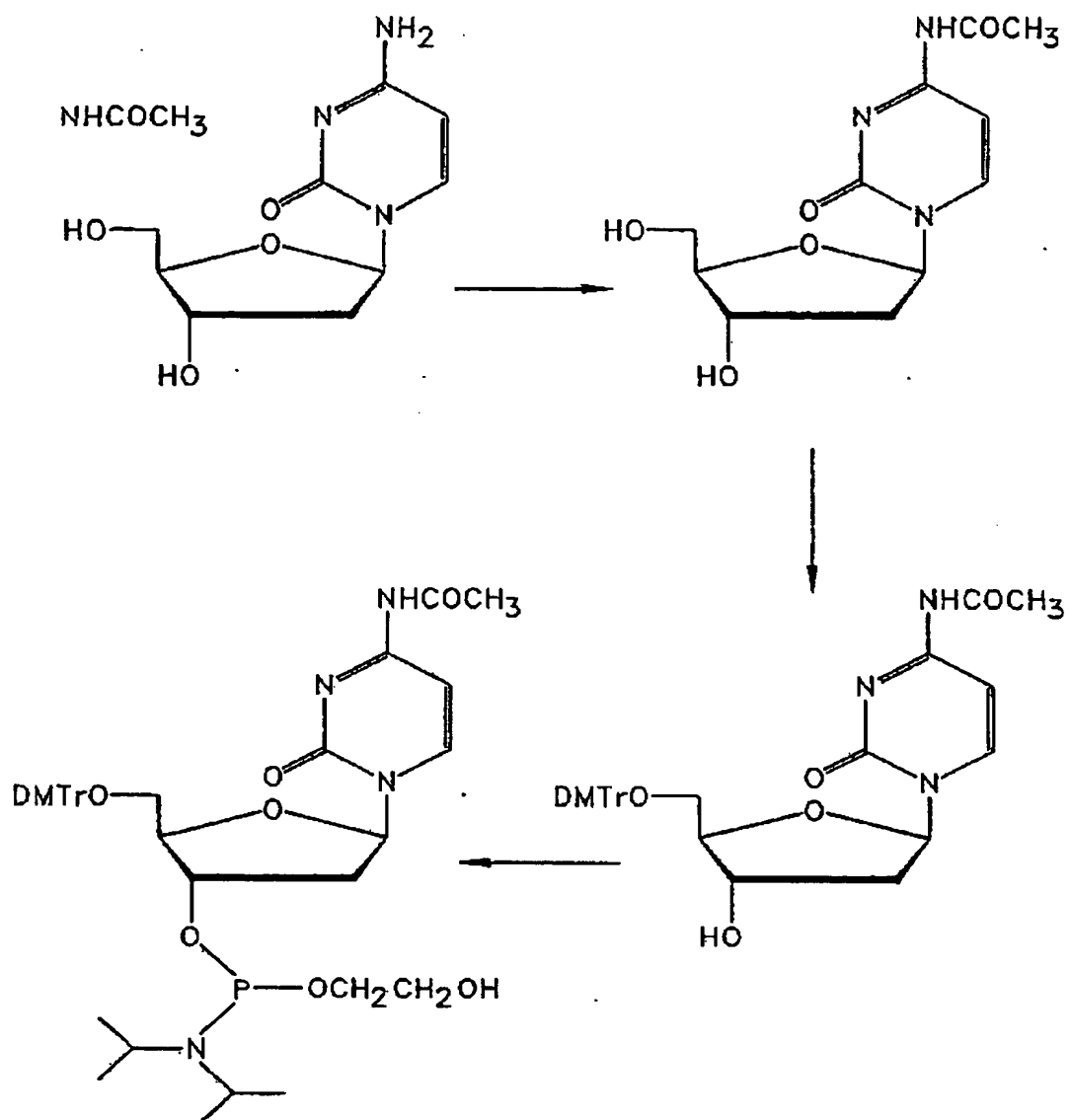


FIG. 1

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9 10 11 12

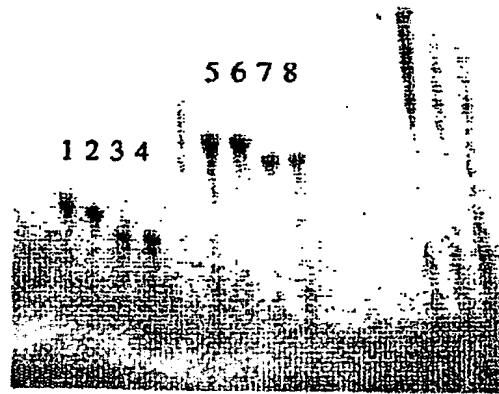


Fig. 2

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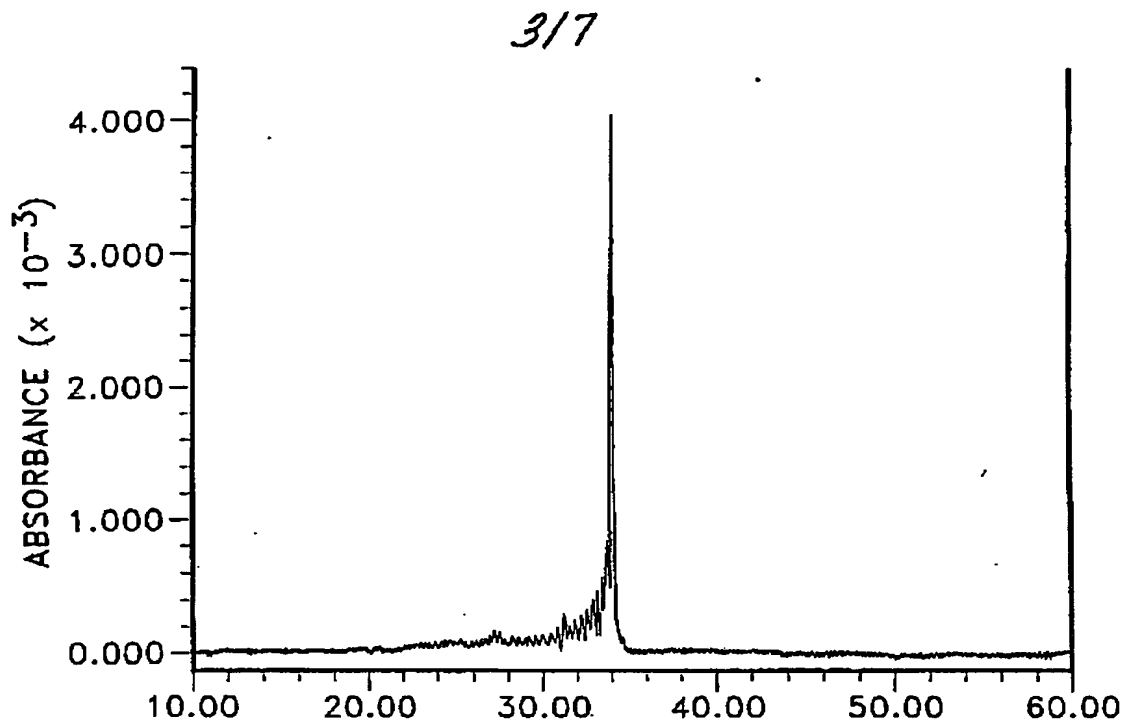


FIG. 3.

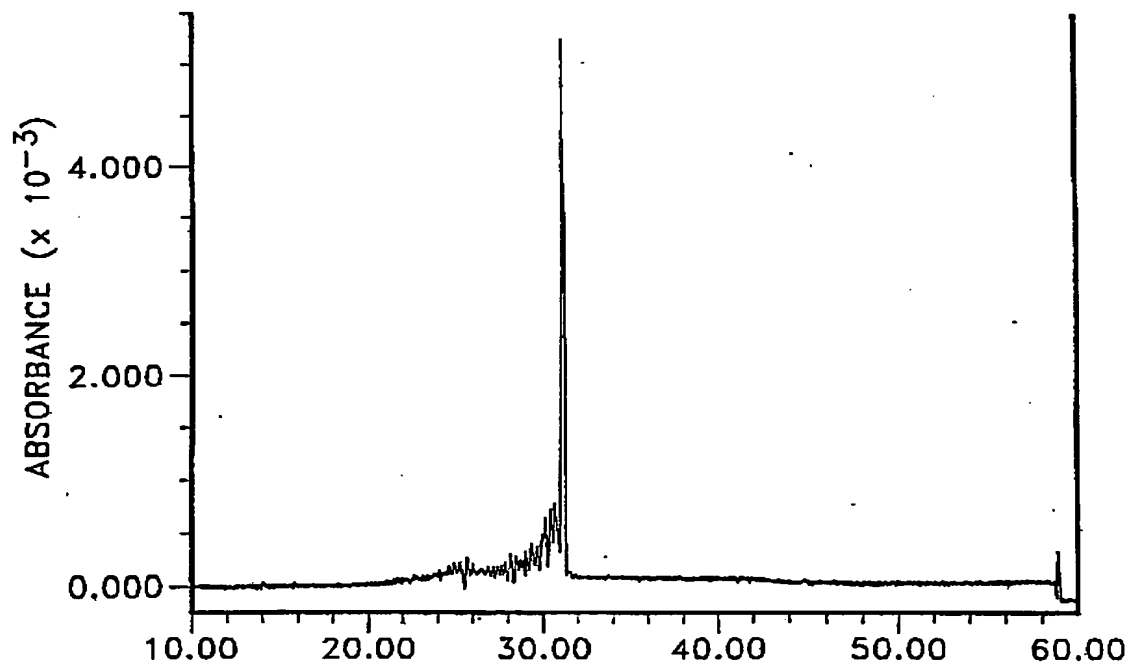
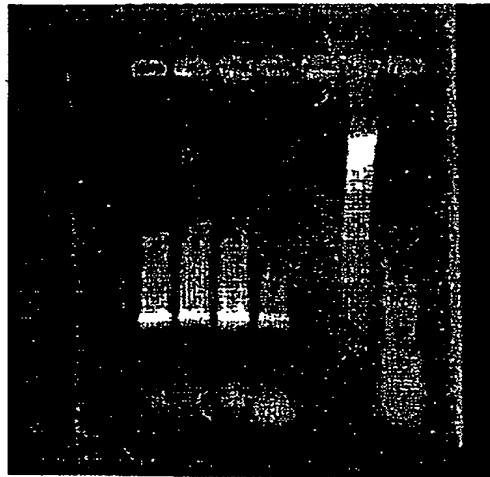


FIG. 4.

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1 2 3 4

Fig. 5

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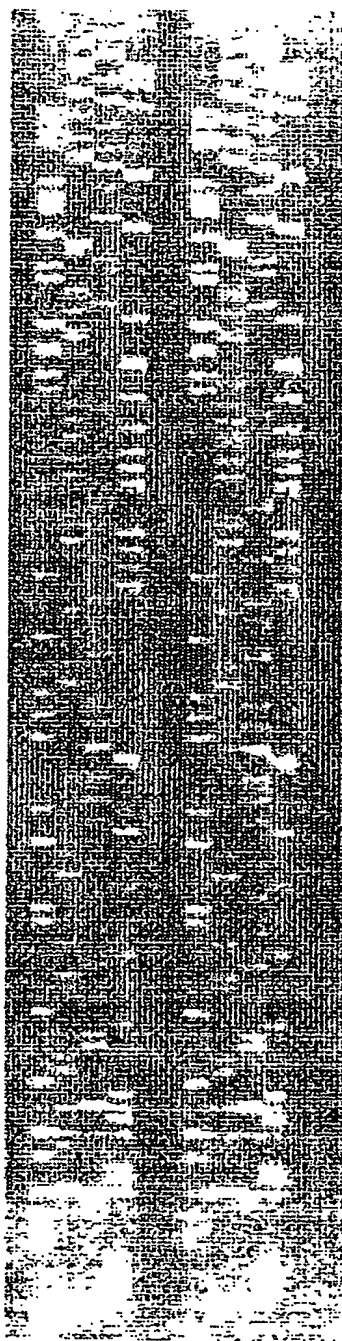


Fig. 6

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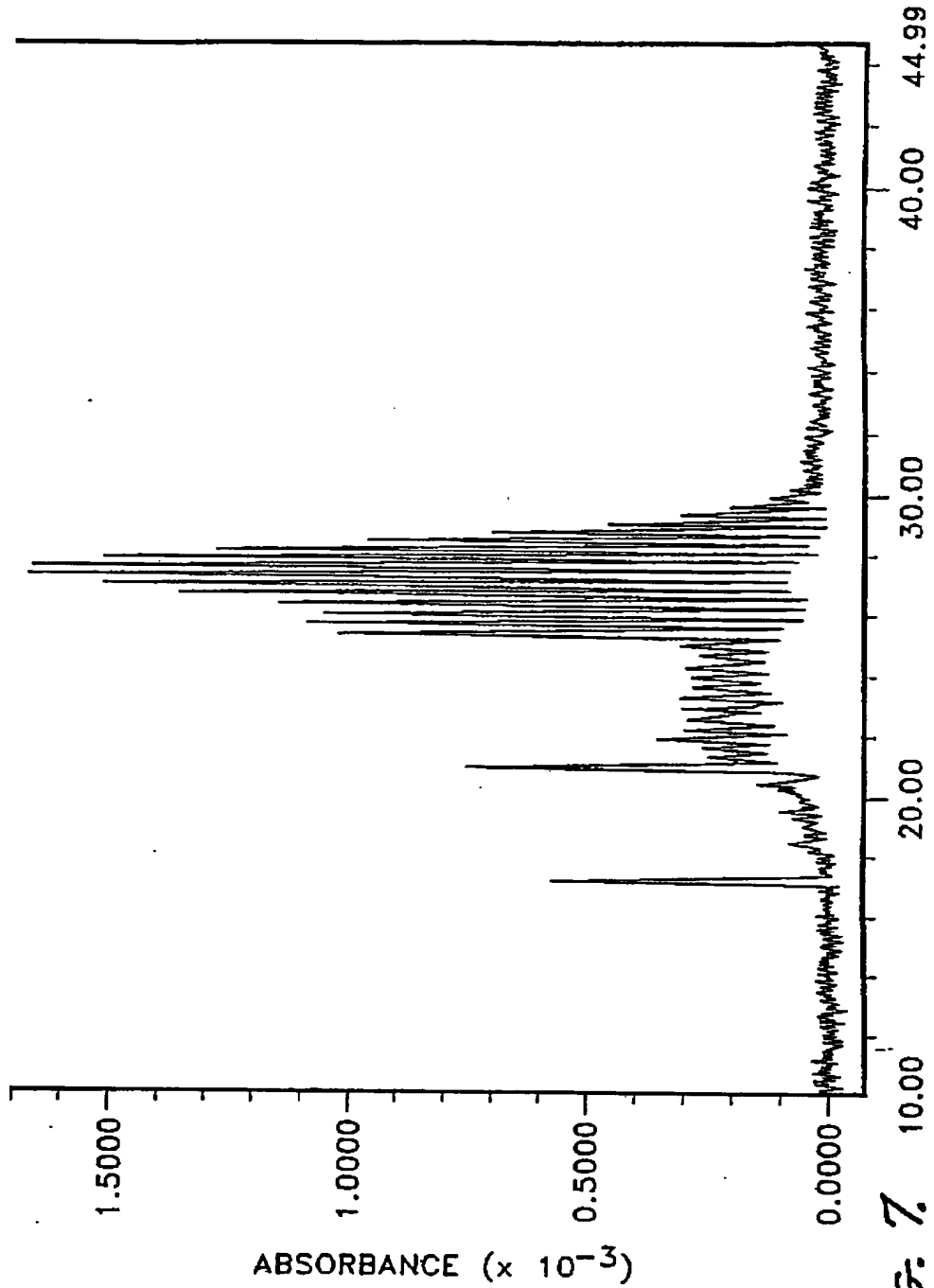


FIG. 7

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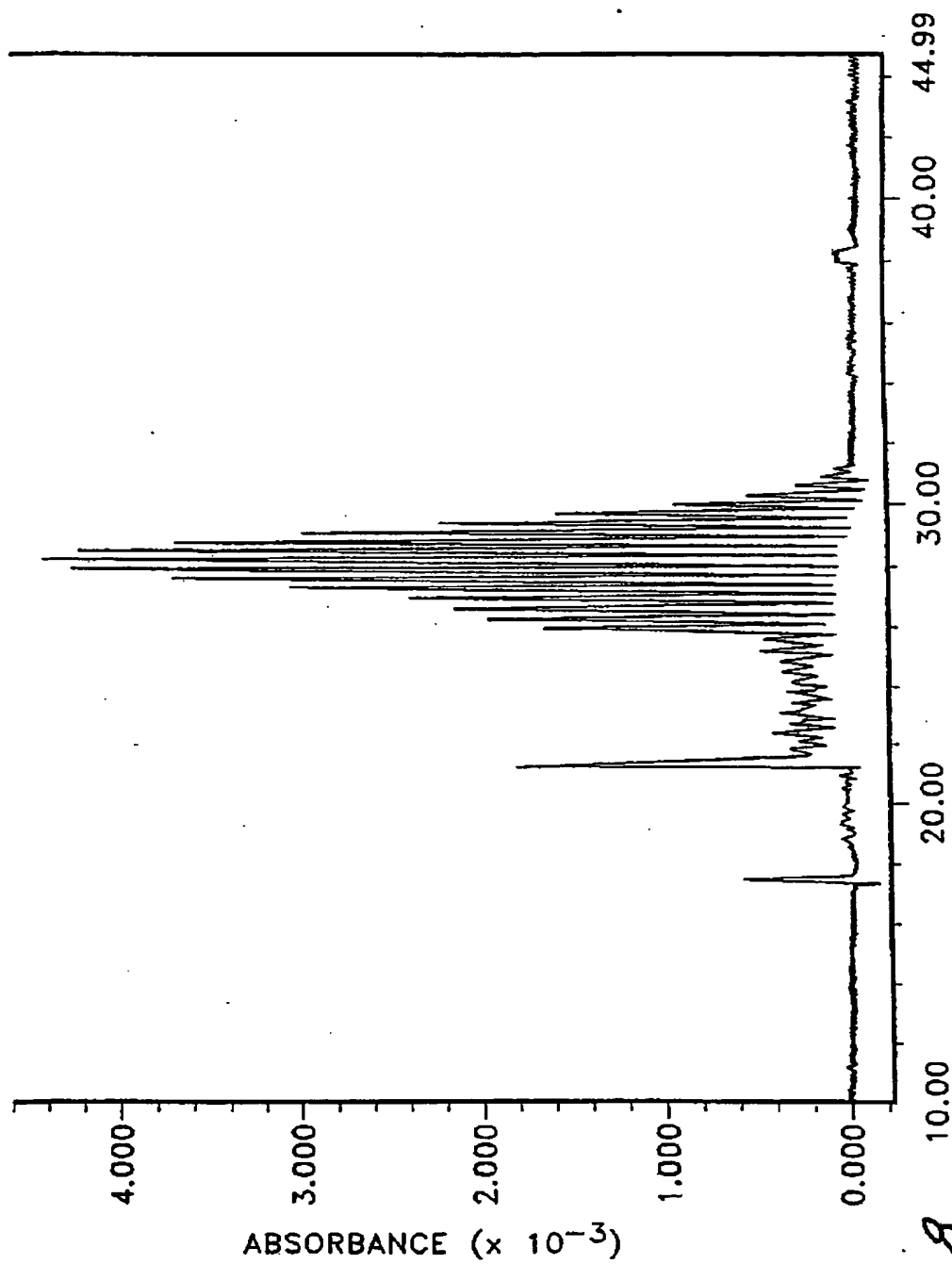


FIG. 8.

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I. CLASSIFICATION OF SUBJECT MATTER		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int.Cl. 5 C07H19/04; C07H21/00		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
Int.Cl. 5	C07H	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹		
Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	WO,A,9 202 535 (APPLIED BIOSYSTEMS INC.) 20 February 1992	1-8
X	JOURNAL OF THE AMERICAN CHEMICAL SOCIETY. vol. 86, 5 August 1964, GASTON, PA US pages 4188 - 4194 LOHRMANN R. AND KHORANA H.G. 'Studies on Polynucleotides. XXXIV. The Specific Synthesis of C3' - C5'-Linked Ribooligonucleotides. New Protected Derivatives of Ribonucleosides and Ribonucleotide 3'-Phosphates. Further Syntheses of Diribonucleoside Phosphates' see page 4188 - page 4189	1-4,9-12
<p>¹⁰ Special categories of cited documents: ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
11 AUGUST 1993	25. 08. 93	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE	DAY G.J.	

Form PCT/ISA/210 (second sheet) (January 1983)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	JOURNAL OF THE AMERICAN CHEMICAL SOCIETY. vol. 83, 5 February 1961, GASTON, PA US pages 686 - 698 KHORANA H.G. ET AL 'Studies on Polynucleotides. IX. Experiments on the polymerization of Mononucleotides. Certain Protected Derivatives of Deoxycytidine-5' Phosphate and the Synthesis on Deoxycytidine Polynucleotides' see page 686 - page 689	1-8
X	JOURNAL OF THE AMERICAN CHEMICAL SOCIETY. vol. 88, no. 4, 20 February 1966, GASTON, PA US pages 819 - 829 LOHRMANN R. ET AL 'Studies on Polynucleotides. LI. Syntheses of the 64 Possible Ribotrinucleotides Derived from the Four Major Ribomononucleotides' see page 819 - page 821	1-4, 9-12
X	JOURNAL OF SCIENTIFIC AND INDUSTRIAL RESEARCH vol. 49, no. 9, September 1990, NEW DELHI pages 441 - 448 SINGH R.K. ET AL 'Protecting groups used in oligonucleotide synthesis: A current survey' see the whole document	1-12
A	WD, A, 8 605 518 (SUMMERTON J. ET AL) 25 September 1986 see page 54, line 31 - line 33; example 19	1

Form PCT/ISA/210 (extra sheet) (January 1985)

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.**

US 9303337
SA 73294

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
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11/08/93

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9202535	20-02-92	None	
WO-A-8605518	25-09-86	AU-A- 5661386	13-10-86
		AU-A- 5698186	13-10-86
		CA-A- 1268404	01-05-90
		DE-A- 3687030	03-12-92
		EP-A, B 0216860	08-04-87
		EP-A- 0215942	01-04-87
		JP-T- 62502338	10-09-87
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		US-A- 5217866	08-06-93

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